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Genetic Diversity and Structure of *Silene kiusiana* (Caryophyllaceae) in the Aso Region, Kyushu, Japan, Revealed by Novel Nuclear Microsatellite Markers

TAKASHI YAMASAKI[1], KEICHI OZEKI[2], NORIYUKI FUJII[1,*], MARI TAKEHARA[3], MASASHI YOKOGAWA[4], SHINGO KANEKO[5] AND YUJI ISAGI[4]

[1]*Graduate School of Science and Technology, Kumamoto University, Kurokami 2-39-1, Chuo-ku, Kumamoto, Kumamoto 860-8555, Japan. *nfujii@kumamoto-u.ac.jp (author for correspondence);* [2]*Nagasaki Prefectural Government, Edo-machi 2-13, Nagasaki, Nagasaki 850-8570, Japan;* [3]*Biodiversity Center of Japan, Ministry of the Environment, Kenmarubi 5597-1, Kamiyoshida, Fijiyoshida, Yamanashi 403-0005, Japan;* [4]*Graduate School of Agriculture, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan;* [5]*Faculty of Symbiotic Systems Science, Fukushima University, Kanayagawa 1, Fukushima, Fukushima 960-1248, Japan*

Silene kiusiana (Caryophyllaceae) is an endangered perennial herb of semi-natural grasslands in Japan and Korea. To elucidate the genetic status of *S. kiusiana* in the Aso region, Kyushu, Japan, in which relatively large populations still remain, we analyzed the genetics of seven populations of *S. kiusiana* using five novel microsatellite markers. Each population of *S. kiusiana* has maintained relatively high genetic diversity (mean H_E = 0.791 and mean N_A = 12.0). In addition, the values of pairwise F_{ST} indicating the degree of genetic differentiation among populations were significantly larger than zero between all pairs of populations, although the degrees of difference were small. The semi-natural grasslands in the Aso region have decreased in size over the past several decades, and thus habitat fragmentation may have caused genetic differentiation among populations of *S. kiusiana*. Although the populations of *S. kiusiana* have maintained relatively high genetic diversity, the genetic dynamics of *S. kiusiana* populations should be continuously monitored.

Key words: Endangered species, Genetic diversity, Genetic structure, Grassland, Habitat fragmentation, Microsatellite, *Silene kiusiana*, The Aso region

The Earth's biodiversity is threatened by human activities (Pimm *et al.* 1995), and many species have already been exterminated (Lawton & May 1995, Regan *et al.* 2001, Smith *et al.* 1993). Of the species that currently exist, many have decreased in population size (Groombridge 1992). If aggressive conservation measures are not taken, about two-thirds of vascular plants may disappear by the end of the 21st century (Pitman & Jørgensen 2002). Approximately 7,000 vascular plant taxa are recognized in the Japanese flora, one-quarter of which are threatened with extinction (Ministry of the Environment of Japan 2007). Conservation practices for these threatened plant species are therefore urgently needed.

Along with habitat destruction and commercial collection, the synergistic destabilizing effects of stochastic processes have been described as an "extinction vortex" (Gilpin & Soule 1986). Some of the stochastic factors are genetic factors, and the decline of genetic variation within populations can cause inbreeding depression and increase the population's risk of extinction (Frankham 2005). It is known that genetic drift reduces the genetic variability of isolated populations and can result in strong genetic differentiation between populations (Lienert 2004). In addition, genetic drift might randomly increase the frequencies of slightly deleterious mutations in a small population, which generally leads to a decrease in variation within the population. In threatened plant species, owing to the reduction of genetic variation within populations, each population often faces an increased short-term risk of extinction because of reduced reproduction due to pollen limitation and inbreeding depression, and an increased long-term risk due to a decreased ability to respond to environmental changes (Frankham *et al.* 2002, Kery *et al.* 2000).

In some endangered plant species, restoration efforts such as introduction of individuals from other populations has been conducted (DeMauro 1993, Hogbin & Peakall 1999, Robichaux *et al.* 1997). Although a specific restoration effort may result in recovery of fitness, or genetic rescue, there is a risk of jeopardizing the evolutionary distinctiveness and the ecological viability of populations due to genetic swamping by the introduced genotypes and by outbreeding depression (Hedrick 2005, Hufford & Mazer 2003). It is thought that populations of most organisms possess an original historical background and may differ from each other in genetic aspects such as genetic diversity and structure. Thus, to conserve endangered species efficiently, it is important to determine the genetic condition of each population or individual, and then plan a conservation strategy based on the results (Neel & Cummings 2003).

Silene kiusiana (Makino) H. Ohashi & H. Nakai (Caryophyllaceae) is an endangered perennial herb of open grassy and swampy fields in mountainous areas in Japan and Korea (Akiyama 2006). It has been reported that its flowering period is from July to September and that the insect-pollinated, protandrous, hermaphroditic flowers ensure cross-pollination (Saito & Sugiura, unpubl.). Our preliminary observations revealed that the inconspicuous seeds, shaped like grains of rice, are 1–1.5 mm long. Although the mode of seed dispersal has not been reported, it is presumed that the seeds are dispersed by gravity. Several other species of *Silene* are reported to have gravity dispersed seeds (Barluenga *et al.* 2011, Young 2002). Traditionally, *Silene kiusiana* has been known as *Lychnis kiusiana* Makino (Kitamura & Murata 1961, Ohwi & Kitagawa 1983), but recent taxonomic studies (Ohashi & Nakai 1996) based on molecular phylogenetic findings (Oxelman & Lidén 1995) have proposed a broader concept of *Silene* to include *Lychnis*, *Melandrium*, and *Cucubalus*. We therefore use the name *Silene kiusiana* in the present study.

Over the last few decades in Japan, changes in rural land use have caused a severe reduction and fragmentation of semi-natural grasslands (Tsukada *et al.* 2004). *Silene kiusiana* is one of several species of such habitats. At present, *S. kiusiana* is known in Japan to occur only in the Aso region, Kyushu, and in the central Chugoku region, Honshu.

Silene kiusiana has significant ornamental value, since it has conspicuous red or pink corollas. The illegal collection of plants is another major factor in the reduction of plant populations and species (Environment Agency of Japan 2000). Based on the observed rapid reduction in the number of remnants and populations, *S. kiusiana* is designated as endangered in the Japanese Red Data List (Ministry of the Environment of Japan 2007).

Although *Silene kiusiana* is at high risk of extinction, the study of the genetics of the species has not been conducted. In the present study, we developed and used microsatellite markers to identify the genetic population structure and diversity in *S. kiusiana*. Highly variable microsatellite markers, which allow the accurate estimation of within- and between-population differentiation, are genetic tools that can be used to detect populations with rare alleles that may be candidates for special protection and populations with low diversity potentially in need of management assistance (Cornuet *et al.* 1999, Estoup *et al.* 1995, Pritchard *et al.* 2000). We sampled extant populations from the Aso region, the remaining relatively large population in Japan. The information obtained will be useful for conservation and restoration plans for *S. kiusiana*.

Materials and Methods

Plant sampling

In July and August 2009–2011, we collected leaves of *Silene kiusiana* from seven populations (MG, OK, NB, TN1, TN2, TN3 and ND) in the Aso region of Kumamoto Prefecture, Kyushu, Japan (Table 1, Fig. 1). Those populations are nearly all that are known from this region. A total of 235 individuals were collected, and sampling was performed randomly throughout each population. All samples were dried and preserved with silica gel. Each population size was estimated roughly according to the number of flowering plants observed.

For the MG population, we established a 7 × 7 m quadrat in which 61 individuals (stems) were mapped and then collected (Fig. 2). The samples were preserved as above and used only to evaluate the fine scale genetic structure (distribution of genets) of *Silene kiusiana.*

Isolation and characterization of microsatellite markers

The development of microsatellite markers in the present study was based on improved technique for isolating codominant compound microsatellite markers (Kaneko *et al.* 2007, Lian & Hogetsu 2002, Lian *et al.* 2006). The restricted DNA library of *S. kiusiana* was constructed by digesting DNA with a blunt-end restriction enzyme,

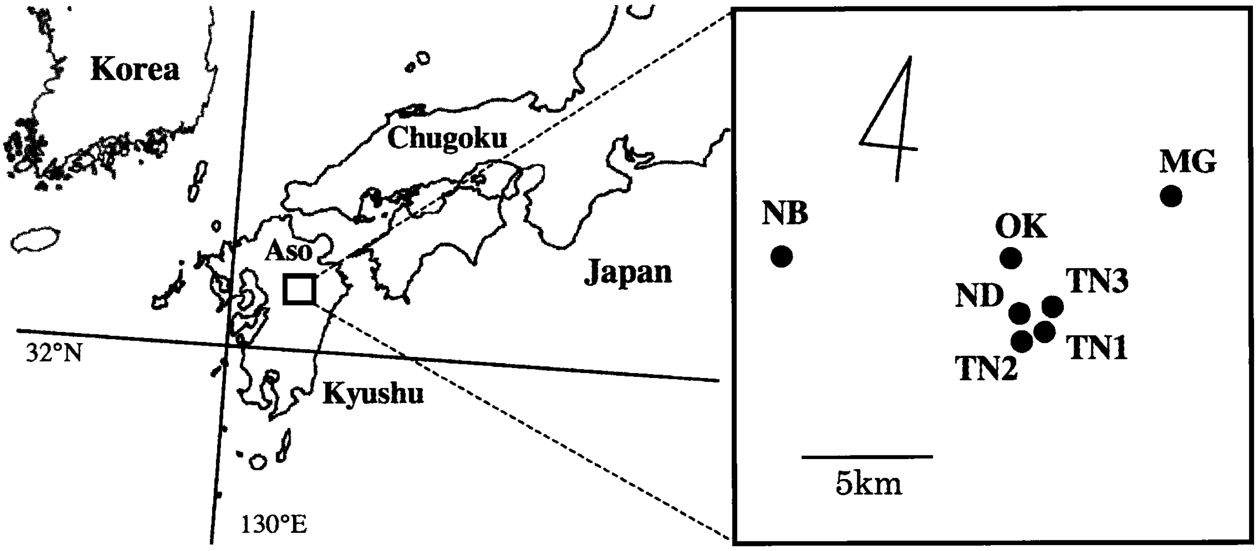

FIG. 1. Sampling locations of *Silene kiusiana* in the Aso region, Kumamoto Prefecture, Kyushu, Japan.

TABLE 1. The materials and their sources analysed for microsatellite variation of *Silene kiusiana* in the Aso region of Kumamoto Prefecture, Kyushu, Japan.

Population	Locality*	Altitude (m)	No. of samples	Estimated population size
MG	Minamioguni	870	50	~1,000
OK	Ichinomiya, Aso	770	39	~1,000
NB	Minamioguni	810	30	~100
TN1	Ichinomiya, Aso	780	20	~200
TN2	Ichinomiya, Aso	780	40	~200
TN3	Ichinomiya, Aso	780	30	~100
ND	Ichinomiya, Aso	780	26	~100
Total			235	

*From a view point of conservation, the information of localities are shown approximately.

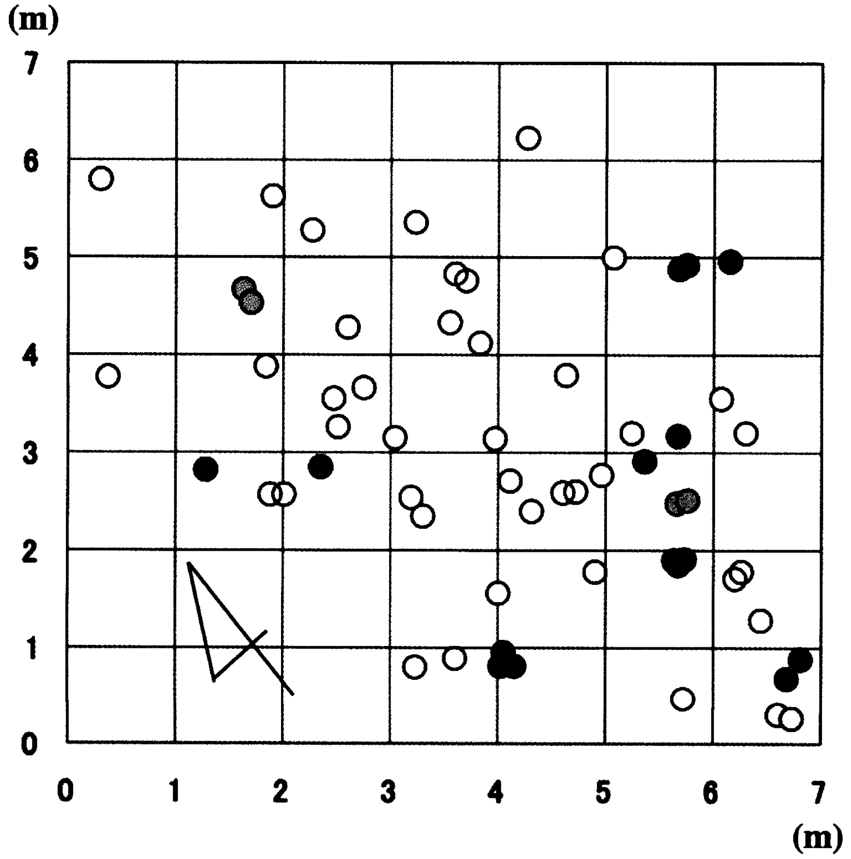

FIG. 2. Distribution of individuals within the quadrat established in the MG population of *Silene kiusiana* (see, Fig. 1 and Table 1). White circles indicate plants that showed a distinct genotype. Same-color circles indicate plants with identical genotypes; we considered these plants clones or same individuals.

*Eco*RV or *Ssp*I. The restriction fragments were then ligated with a specific blunt adaptor (consisting of the 48-mer: 5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3' and an 8-mer with the 3'-end capped with an amino residue: 5'-ACCAGCCC-NH_2-3') using the DNA ligation kit (Takara Bio). Fragments were amplified from the *Ssp*I DNA library using compound microsatellite primer $(AC)_6(AG)_7$, $(AC)_6(TC)_5$, or $(AC)_6(AG)_5$ and an adaptor primer (5'-CTATAGGGCACGCGTGGT-3'). The amplified fragments, ranging from 400 to 800 bp, were then separated on a 1.5% LO3 agarose gel (Takara Bio) and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified DNA fragments were subsequently cloned using the QIAGEN PCR Cloning plus Kit (Qiagen) according to the manufacture's instruction. Briefly, polymerase chain reaction (PCR) products were ligated into the pDrive vector and transformed into QIAGEN EZ competent cells. The cloned fragments containing an $(AC)_6(TC)_n$, $(AC)_6(AG)_n$, or $(AG)_6(AC)_n$ compound microsatellite sequence were amplified using M13 forward and reverse primers from the plasmid DNA of the positive clones. Based on the sequences obtained, 28 candidate primers were designed using Primer3 ver. 0.4.0 (Rozen & Skaletsky 2000). Out of the candidate loci, four loci (*Ski145, Ski221, Ski366* and *Ski461*) were well amplified by PCR and showed polymorphic band patterns within and among the populations (Table 2). In addition, one marker (*Ssi226*) developed by the same protocol in *Silene sieboldii* (Van Houtte) H.Ohashi & H.Nakai (Yokogawa *et al.* unpubl.) was successfully amplified and showed polymorphic band patterns as with the above four markers. We then checked the efficiency of the primers using the 50 plants from the MG population (not the samples for fine scale analysis). The linkage disequilibrium test using FSTAT version 2.9.3.2 (Goudet 1995) found no significant genotype disequilibrium between all pairs of loci ($P > 0.05$). Additionally, no significant departure from Hardy-Weinberg equilibrium (HWE) was detected in all loci through Bonferroni correction with a 0.05 significance threshold. Thus, these five loci were used for further analysis.

Microsatellite genotyping

Genomic DNAs of all samples were extracted from leaves by modified version of CTAB method (Doyle & Doyle 1987). The five microsatellite loci as mentioned above were amplified by PCR using a QIAGEN Multiplex PCR Kit (Qiagen) (Table 2). The 5 μL PCR reaction mixtures contained 0.5 μL template DNA (approx. 1–5 ng), 1.0 μM each primers, and Master mix 2.5 μL. The amplification profiles included initial denaturation at 95°C for 15 min, followed by 30 cycles of 30 s at 94°C, 90 s at a specific annealing temperature of primers (Table 2), 1 min at 72°C; then final extension at 60°C for 30 min. The reaction was carried out with the Thermalcycler 2720 (Applied Biosystems). The size of the PCR products was estimated using the CEQ 8000 Analysis System (Beckman Coulter) with a DNA Size Standard Kit-400 (Beckman Coulter).

Data analysis

We sampled and genotyped 235 plants from seven populations (Table 1). To estimate genetic diversity within each population, the mean number of alleles per locus (N_A), the expected and observed heterozygosity (H_E and H_O), and the number of private alleles (P_R) that are only present in a single population were calculated using GenAlEx version 6.2 (Peakall & Smouse 2006). Also, allelic richness (A_R; El Mousadik & Petit 1996) and inbreeding coefficients (F_{IS}) were calculated using FSTAT ver. 2.9.3.2. The latter parameter, F_{IS}, was tested with 700 randomizations and Bonferroni correction to determine whether it differed significantly from zero.

The analysis of molecular variance (AMOVA) was carried out using GenAlEx ver. 6.2 to estimate the levels of genetic variation among and within populations. The pairwise F_{ST} (Weir & Cockerham 1984) was computed using FSTAT ver. 2.9.3.2 to reveal the degree of genetic differentiation among populations, and was also tested with 420 permutations to determine whether it differed significantly from zero. To test whether genetic differentiation among populations followed an isolation by distance pattern, a Mantel test (Mantel 1967) was conducted using GenAlEx ver. 6.2. In this test, the latitude and the longitude data of each population were used to determine geographic distance, and $F_{ST} / (1-F_{ST})$ between populations was used to determine genetic distance. The relationship among populations was inferred using a neighbor joining (NJ) tree based on genetic distance among populations D_A (Nei *et al.* 1983), generated by Population 1.2.30 beta (Langella 2007). The robustness of each branch of the tree was evaluated by bootstrap probability from 1,000 resamplings of the genotype dataset. To infer the genetic structure of populations, a Bayesian clustering was implemented using STRUCTURE version 2.3.3 (Pritchard *et al.* 2000). This approach estimates the number of clusters (K) and assigns each individual to these clusters. We used an admixture model, an allele frequencies correlated model (F-model; Falush *et al.* 2003) and the LOCPRIOR model (Hubisz *et al.* 2009). The F-model assumes that all clusters diverged from a common ancestral population and considers that each of the clusters may have experienced a different degree of genetic drift (Falush *et al.* 2003). In this model, the amount of genetic drift for each cluster is described as the F-value. The LOCPRIOR model modifies the prior distribution for each individual's population assignment and allows the proportion of individuals assigned to particular clusters to vary by location (Hubisz *et al.* 2009). A series of 20 independent runs for each value of K ranging from 1 to 8 was performed with 1×10^6 MCMC iterations after 1×10^5 burn-in periods. To identify the appropriate value of K, we used two methods. In the first method, we compared the mean value of log probability of data (Ln

$P(D)$) for each K according to Pritchard *et al.* (2000). In the second method, we calculated the ad hoc statistic using Ln $P(D)$, ΔK in successive K values according to Evanno *et al.* (2005).

Estimation of the fine-scale genetic structure of S. kiusiana

To estimate the fine-scale genetic structure (distribution of genets) of *S. kiusiana*, we genotyped all individuals collected from the 7 × 7 m quadrat established in the MG population using the five microsatellite loci and methods described above. We calculated the probability of identity, which estimates the average probability that two unrelated individuals drawn from the same randomly mating population will by chance have the same multilocus genotype, using GenAlEx ver. 6.2. Because the probability for the five loci was very small (2.91×10^{-8}), the plants with identical multilocus genotypes were regarded as clones or same individuals. We investigated the distribution of the genets according to the location in the quadrat and genotype of each individual.

Results

Marker loci polymorphism

The number of alleles per locus ranged from four for *Ssi226* to 29 for *Ski145*, with an average of 21.8 (Table 2); a total of 109 alleles were detected. The observed and expected heterozygosities (H_O and H_E) ranged from 0.468 for *Ssi226* to 0.906 for *Ski461* and from 0.579 for *Ssi226* to 0.941 for *Ski461*, with averages of 0.759 and 0.840, respectively.

Genetic diversity within populations

The mean number of alleles across loci (N_A) ranged from 9.0 for ND to 14.6 for TN2, and the allelic richness (A_R) ranged from 8.3 for ND to 11.8 for TN2; the mean values were 12.0 and 10.4, respectively (Table 3). Out of 109 total alleles 21 were private (observed in the single population). The mean number of private allele per locus ranged from 0.2 for NB and TN1 to 1.0 for OK, TN2 and ND, with an average of 0.6. Although each population size was different, the heterozygosities of all populations showed similar values. H_O ranged from 0.654 for ND to 0.850 for TN1 (mean, 0.756), H_E ranged from 0.752 for ND to 0.825 for TN2 (mean, 0.791). F_{IS} ranged from −0.052 for TN1 to 0.150 for ND, and the higher inbreeding coefficient significantly different from zero was observed in the NB population ($P < 0.05$).

Genetic differentiation and structure among populations

Although the degree of geographical distance among populations was small (0.7–15.1 km), significant genetic differentiation was observed. The variance test (AMOVA) showed that 9% of the to-

TABLE 2. Characteristics of the five compound microsatellite loci for 235 samples of *Silene kiusiana* and their variability.

Locus	Repeat motif	Primer sequence (5'-3')	T_a (°C)	Size range (bp)	A	H_E	H_O	Accession No.
Ski145	$(AC)_6(TC)_{19}$	ACACACACACACTCTCTCTCTC AGAGGGTGGACAACGAACAT	57	117–177	29	0.932	0.902	AB710380
Ski221	(AC)6(TC)14	ACACACACACACTCTCTCTCTC GCGGGAGAGTAAATGAAAG	57	84–136	24	0.818	0.757	AB710381
Ski366	$(AC)_6(AG)_{19}$	ACACACACACACAGAGAG AGTACTTATGAGACGGTTTA	49	131–185	26	0.931	0.762	AB710382
Ski461	$(AC)_6(TC)_{17}$	ACACACACACACTCTCTCTCTC ATTTGGCTTTCACCTTATGTT	57	212–266	26	0.941	0.906	AB710383
Ssi226	$(AG)_6(AC)_9$	AGAGAGAGAGAGACACACACAC TCTCCCGTGTCTGAGTAACATA	57	122–128	4	0.579	0.468	AB478867
Mean					21.8	0.840	0.759	

T_a, annealing tempetature; A, the number of allele; H_E, expected heterozygosity; H_O, observed heterozygosity

TABLE 3. Genetic diversity within populations of *Silene kiusiana*.

Population	N_A	A_R	P_R	H_O	H_E	F_{IS}
MG	14.2	11.5	0.4	0.808	0.817	0.021
OK	13.0	10.7	1.0	0.774	0.756	−0.010
NB	12.4	11.0	0.2	0.713	0.809	0.135*
TN1	9.8	9.8	0.2	0.850	0.789	−0.052
TN2	14.6	11.8	1.0	0.765	0.825	0.085
TN3	10.8	9.8	0.4	0.727	0.792	0.099
ND	9.0	8.3	1.0	0.654	0.752	0.150
Mean	12.0	10.4	0.6	0.756	0.791	

N_A, mean number of alleles per locus; A_R, number of private alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient. *Significant deviation of F_{IS} value from zero tested with 700 randomizations ($P < 0.05$).

tal genetic variation existed among populations of *S. kiusiana*, and the remaining genetic variations were within populations (Table 4). Pairwise F_{ST} ranged from 0.016 between the NB and TN2 populations to 0.112 between the OK and ND populations, and the F_{ST} values were significantly larger than zero ($P < 0.05$) between any pairs of populations (Table 5).

The analysis of genetic structure consistently showed that genetic differentiation among populations did not correspond to geographical distribution. The Mantel test did not indicate a positive correlation between geographic distance and genetic distance among populations ($R^2 = 0.009$, $P = 0.435$). The neighbor-joining tree based on genetic distance D_A among populations showed that the TN1 and ND populations were clustered with 98% bootstrap probability, and the NB and TN2 populations formed a cluster supported by 79% bootstrap probability (Fig. 3). In the Bayesian clustering, the mean log probability of the data (Ln $P(D)$) over 20 runs for each cluster was the highest at $K = 6$ (Fig. 4). The result of a single run at $K = 6$ is shown in Fig. 5a. The MG and OK populations belonged to clusters 1 and 2, respectively. The NB and TN2 populations were assigned to cluster 3 and TN1 was assigned to cluster 4 with high proportion. In the TN3 and ND populations, the clusters of highest proportion of membership were clusters 5 and 6, respectively.

TABLE 4. Analysis of molecular variance (AMOVA) for 235 individuals in seven populations of *Silene kiusiana*.

Source of variance	*df*	Sum of squares	Mean squares	Variance component	Total variance (%)
Among populations	6	106.852	17.809	0.408	9
Within populations	228	980.016	4.298	4.298	91

TABLE 5. Pairwise F_{ST} (below diagonal) and geographical distance (km; above diagonal) among seven populations of *Silene kiusiana*.

	MG	OK	NB	TN1	TN2	TN3	ND
MG		6.4	15.1	7.4	8.1	6.6	7.4
OK	0.039*		9.1	3.3	3.4	2.6	2.5
NB	0.044*	0.031*		10.8	10.3	10.8	10.0
TN1	0.054*	0.064*	0.032*		0.7	0.9	0.9
TN2	0.054*	0.059*	0.016*	0.031*		1.4	1.0
TN3	0.042*	0.034*	0.019*	0.031*	0.030*		0.9
ND	0.079*	0.112*	0.072*	0.046*	0.057*	0.068*	

*Significant differences from zero are indicated ($P < 0.05$).

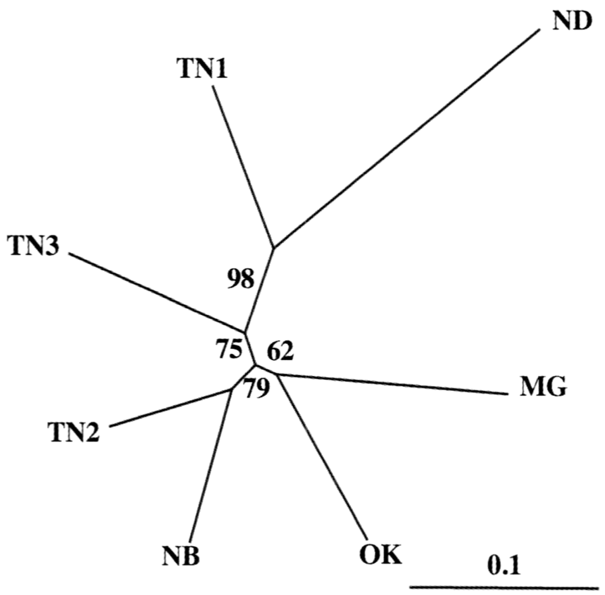

FIG. 3. Neighbor joining (NJ) tree based on genetic distance (D_A) among the seven populations of *Silene kiusiana*. The numbers along the branches are bootstrap values (%) based on 1,000 replicates. For the population name abbreviations, see Table 1.

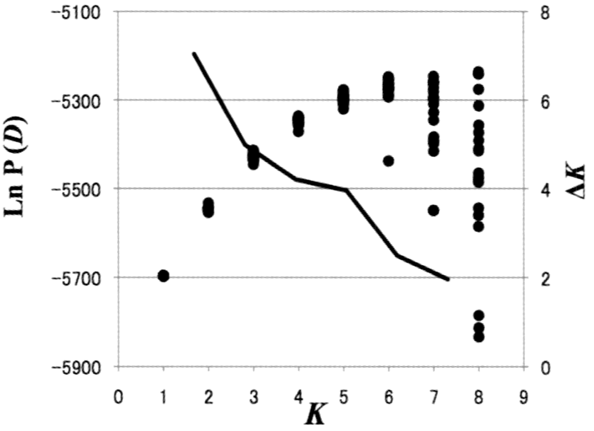

FIG. 4. Distribution of the values of log probability of data (Ln $P(D)$) for each cluster (K) ranging from 1 to 8 (primary y-axis) and ΔK based on the Ln $P(D)$ between successive K-values (secondary y-axis).

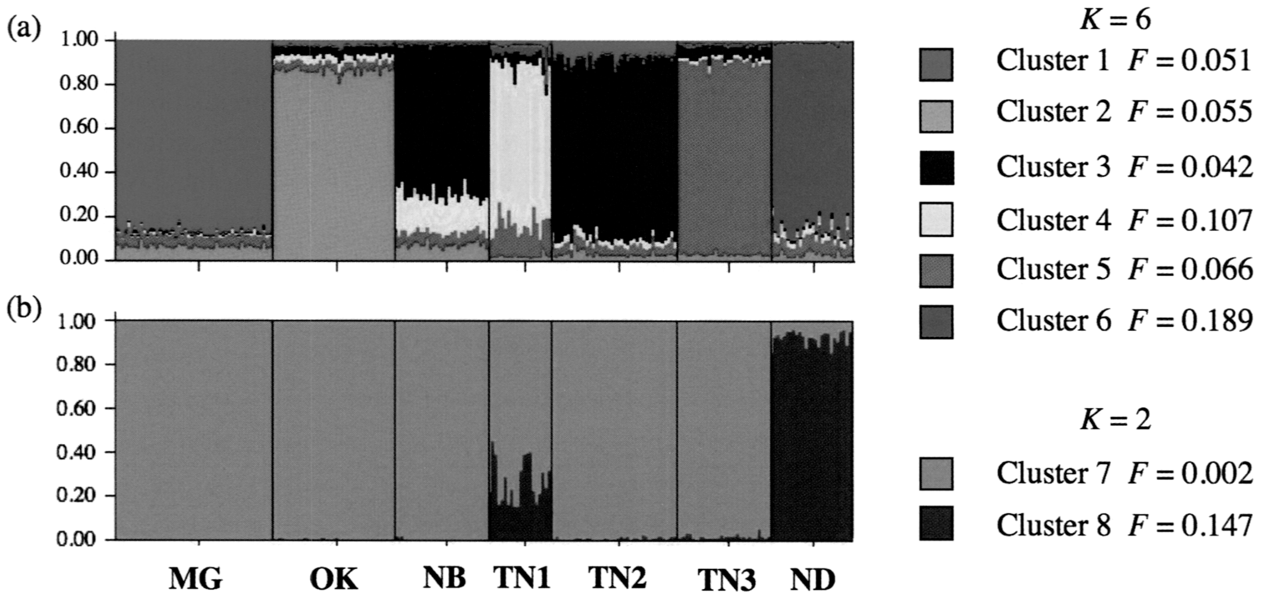

FIG. 5. The proportion of the membership of clusters in 235 individuals from seven populations of *Silene kiusiana*. (a) The optimal K-value (6) was determined by Bayesian clustering according to Pritchard *et al.* (2000); (b) the value (2) was estimated by calculating ΔK according to Evanno *et al.* (2005).

The F-value of each cluster ranged from 0.042 to 0.189, the highest value of which was in cluster 6. When we used the method described by Evanno *et al.* (2005), the value of ΔK was the highest at $K = 2$ (Fig. 4). The ND population and part of the TN1 population were assigned to cluster 8, whereas the others were in cluster 7 (Fig. 5b). The F-value of cluster 7 was 0.002, and that of cluster 8 was 0.147.

Quadrat investigation in the MG population

All individuals (61 ramets) in the quadrat established in the MG population were composed of 48 genets, based on the multilocus genotypes of the five microsatellite markers (Fig. 2). The largest number of ramets per genet was four (Fig. 2, the blue circles). The longest distance between plants of the same genet was 107 cm (orange circles), and the shortest was 5 cm (blue circles). The shortest distance between plants of different genets was 9 cm.

Discussion

High genetic diversity in Silene kiusiana

Microsatellite markers show high species-specificity, because they have high rates of mutation (e.g., Dallas 1992, Weber & Wong 1993). It is therefore difficult to strictly compare the values of genetic diversity among different species based on this marker. We found—although it is a rough comparison—that the genetic diversity within populations of *S. kiusiana* determined in this study was relatively high in comparison with the related species, *Silene flos-cuculi* (L.) Clairv., and to two other endangered species in the Aso region of Kyushu, Japan; *Echinops setifer* Iljin (Asteraceae) and *Primula sieboldii* E. Morren (Primulaceae). In *S. flos-cuculi*, a perennial plant in wetlands and widely distributed in Europe, the parameters of genetic diversity were H_E = 0.83, determined from seven microsatellite loci in 28 populations in Switzerland (Galeuchet *et al.* 2005). In *E. setifer*, designated as a vulnerable species in Japan (Ministry of the Environment of Japan 2007), H_E = 0.33 was determined using eight microsatellite loci from 10 populations in the Aso region (Kaneko *et al.* 2009). In *P. sieboldii* as well as in *E. setifer*, designated as vulnerable species in Japan, the parameter of genetic diversity of the Aso population was H_E = 0.786 by using eight microsatellite loci (Honjo *et al.* 2009). Moreover, Nybom (2004) reported that the mean H_E values in short-lived perennial, narrowly distributed, outcrossing plants were 0.55, 0.56 and 0.65, respectively, based on microsatellite markers. In the present study, *S. kiusiana* showed H_E = 0.791 (Table 3), indicating a relatively high incidence genetic variation compared to other species with similar life-history attributes, although the sizes of the populations of *S. kiusiana* are currently decreasing (Environmental Agency of Japan 2000).

The system of sexual reproduction is mentioned as one of the factors leading to relatively high genetic diversity, as shown with *S. kiusiana*. It is known that the reproductive system influences genetic diversity within species (Hamrick & Godt 1996). The quadrat investigation in the present study revealed that most of the individuals showed distinct genotypes (Fig. 2), suggesting that sexual regeneration is predominant in this species. Although we detected several clumps composed of two to four individuals with the same genotype (possible genets), the longest distance between plants of the same genet was 107 cm (orange circles) and thus vegetative reproduction is limited. There is also circumstantial evidence that some species of *Silene* do not spread clonally (Menges & Dolan 1998, Tero *et al.* 2003). Moreover, in *S. kiusiana*, it has been reported that the flowers are hermaphroditic and protandrous (Saito & Sugiura, unpubl.), thereby promoting outbreeding due to protandry (Bertin & Newman 1993, Webb & Lloyd 1986). These characteristics may explain the relatively high genetic diversity in the species.

Another possible explanation for the relatively high genetic diversity in *S. kiusiana* are the demographic processes (population size changes) in the Aso region. It was reported that the semi-natural grassland area of the Aso region has decreased from approx. 70,000 ha in 1900 to approx. 36,000 ha around 1990 (National Parks Association of Japan 1995, Shoji 2006). The loss of the semi-natural grassland area has accelerated in the recent two decades. At present, the area has decreased to 23,000 ha (Takahashi 2010). With this reduction, the wetland area, the main habitat of *S. kiusiana*, has also decreased (Sato 2001). We therefore surmise that the size of the populations of *S. kiusiana* have decreased correspondingly. In small fragmented populations, random genetic drift will reduce genetic diversity. However, several generations will be required before genetic drift has a significant impact, especially in long-lived perennial herbaceous plants (Ohara *et al.* 2006, Tomimatsu & Ohara 2003). Although it is not clear that *S. kiusiana* is long-lived, it has been reported that survivorship in adults of *Silene* is high (Kephart & Paladino 1997, Menges & Dolan 1998). Hence, although severe habitat reduction has occurred, the genetic diversity of *S. kiusiana* may be maintained at a high level.

The inbreeding coefficient (F_{IS}) in the NB population was significantly larger than zero. While the ND population showed a relatively high value of F_{IS}, it was not significant (Table 3), indicating that inbreeding may have occurred in these populations. It should be noted that the NB and ND populations are among the smallest examined in this study (Table 1). Within a small population, inbreeding is likely to occur due to the limited number of reproductive partners, and this increases the extinction risk within the population (Frankham 2005, Frankham *et al.* 2002). The estimated size of most populations of *S. kiusiana* is approx. 100 to 200 individuals (Table 1), thus it is considered a high probability that inbreeding reproduction occurs in most populations in the Aso region.

Genetic structure and habitat fragmentation

The value of pairwise F_{ST} indicating the degree of genetic differentiation among populations of *S. kiusiana* was significantly larger than zero between all pairs of populations (Table 5). In addition, Bayesian clustering according to Pritchard *et al.* (2000) showed the presence of six clusters within the species (Fig. 5a). Although in the NB and TN2 populations the proportion of membership of identical clusters was large, most of the individuals in the other five populations were included in different clusters. Genetic differentiation is therefore considered to have occurred among the populations of *S. kiusiana.*

Over the past several decades, as mentioned above, the habitat of *Silene kiusiana* (and other grassland plants) has decreased through succession, or by desiccation of wetlands, accompanied by a decline in semi-natural grassland areas in the Aso region (Sato 2001). Within this region, wetlands are distributed discontinuously along streams among gentle hills. Due to the loss of wetlands, remnant habitats may have become more isolated. Since modes of reproduction affect genetic differentiation among populations, the system of pollination and seed dispersal should be considered in *S. kiusiana.* It is generally believed insect pollinated species with gravity seed dispersal have less gene flow between populations in comparison with wind pollination and animal or wind dispersed seeds (Loveless and Hamrick 1984). Although the pollinators of *S. kiusiana* are unknown, it has been reported that most species of *Silene* are pollinated by insects (moths, butterflies, bumblebees, etc.) (Dötterl *et al.* 2006). Furthermore, *S. kiusiana* has small seeds (ca. 1–1.5 mm long). We surmise that the seeds are dispersed by gravity, as reported for several other species of *Silene* (Barluenga *et al.* 2011, Young 2002). *Silene kiusiana* is probably insect pollinated and has gravity dispersed seeds, making it susceptible to the influence of habitat fragmentation.

According to Nybom (2004), the mean F_{ST} value of 33 published studies, which excluded vegetatively reproducing or apomictic species, based on microsatellite markers was 0.26. In the present study, the F_{ST} values were relatively small (0.016–0.112), although the pairwise F_{ST} between all pairs were significantly larger than zero (Table 5). Moreover, the AMOVA showed only 9% genetic variation among populations of *S. kiusiana* (Table 4). The Bayesian clustering according to Evanno *et al.* (2005) showed that there is almost no genetic differentiation among populations other than the ND population (Fig. 5b). Thus the degree of genetic differentiation among populations in *S. kiusiana* may be relatively small.

This tendency has been observed in other studies of *Silene.* Moyle (2006) compared the pattern of genetic differentiation (F_{ST} and related statistics) among 17 congeneric species of *Silene* and showed that the degree of differentiation is likely to be smaller when the spatial scale of the investigated populations is less than 10 km, although endemic or narrowly distributed species tend to show larger genetic variation among populations. There have also been several reports that the degree of genetic differentiation was small after habitat fragmentation in plants in grasslands when the geographic distance between populations was less than 20 km (e.g., Pluess & Stocklin 2004, Van Rossum *et al.* 2004, Leimu & Mutikainen 2005). In the present study, the most distant populations of *S. kiusiana* were between the NB and MG populations (approx. 15 km) in the Aso region (Table 5). Therefore, the relatively small genetic divergence among the populations of *S. kiusiana* may be related to its narrow range of distribution.

In the Aso region, small genetic differentiation among populations of *S. kiusiana* was inferred. In addition, the Mantel test did not show a pattern of isolation by distance. Furthermore, the NJ tree showed that very distant populations belonged to the same cluster (i.e., the NB and TN2 populations) (Figs. 1, 3) whereas populations that were close to each other (TN1, TN2 and TN3) did not belong to the same clusters. These finding suggest that the populations of *S. kiusiana* in the Aso region do not have a clear geographic genetic structure. These findings may be related to the historical shallowness of habitat fragmentation and the narrow distribution area of *S. kiusiana* in the Aso region.

Conservation perspective

We investigated the genetic diversity of *S. kiusiana* from seven populations, which are nearly all the populations known in the Aso region. Although it was revealed that each population of *S. kiusiana* has maintained relatively high genetic diversity (Table 3), small fragmented populations are subject to extinction or substantial reduction in population size from stochastic events (Lande 1993, Melbourne & Hastings 2008), which could lead to genetic erosion depending upon the severity of reduction of the effective population size. The populations of *S. kiusiana* in the Aso region are evidently small (estimated roughly as 100–1000 individuals/population) (Table 1). In fact, the *F*-values in the ND and TN1 populations (Cluster 4 for 0.107, Cluster 6 for 0.189 and Cluster 8 for 0.147) were higher than in other populations in the Bayesian structuring analyses (Fig. 5). The findings suggest that both populations experienced severe genetic drift and bottlenecks following their divergence from a common ancestral population than did other populations. Furthermore, it was suggested that the inbreeding coefficients (F_{IS}) were relatively high in the ND and NB populations (Table 3). Continuous monitoring should therefore be carried out on the population dynamics of *S. kiusiana.*

Significant genetic differentiation among populations was observed between all pairs of populations in the Aso region (Table 5), presumably due to habitat fragmentation in recent years. The degree of genetic differentiation was small, however, and each population contained relatively high genetic diversity. Although some studies indicate that genetic input from other populations is beneficial (Van Rossum 2008, Willi *et al.* 2005), our findings suggest that genetic rescue is not yet necessary for *S. kiusiana*. Instead, we should make an effort to maintain the semi-grasslands (including wetlands) in the Aso region and increase the population size of *S. kiusiana* without risking genetic incompatibility associated with inter-populational seed transfer.

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